

Does vitamin C have the ability to augment the therapeutic effect of bone marrow-derived mesenchymal stem cells on spinal cord injury?

Nesrine Salem¹, Mohamed Y. Salem¹, Mohammed M. Elmaghrabi², Moataz A. Elawady², Mona A. Elawady³, Dina Sabry^{4, *}, Ashraf Shamaa⁵, Abdel-Haleem H. Elkasapy⁶, Noha Ibrhim⁷, Azza Elamir⁸

1 Department of Histology and Cell Biology, Faculty of Medicine, Banha University, Banha, Egypt

2 Department of Neurosurgery, Faculty of Medicine, Banha University, Banha, Egypt

3 Department of Community Medicine, Faculty of Medicine, Banha University, Banha, Egypt

4 Department of Medical Biochemistry, Faculty of Medicine, Cairo University, Giza, Egypt

5 Department of Surgery and Radiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt

6 Department of Surgery, Faculty of Veterinary Medicine, Banha University, Banha, Egypt

7 Department of Medical Physiology, Faculty of Medicine, Banha University, Banha, Egypt

8 Department of Medical Biochemistry, Faculty of Medicine, El Fayoum University, Egyptian, Egypt

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Abstract

Methylprednisolone (MP) is currently the only drug confirmed to exhibit a neuroprotective effect on acute spinal cord injury (SCI). Vitamin C (VC) is a natural water-soluble antioxidant that exerts neuroprotective effects through eliminating free radical damage to nerve cells. Bone marrow mesenchymal stem cells (BMMSCs), as multipotent stem cells, are promising candidates in SCI repair. To evaluate the therapeutic effects of MP, VC and BMMSCs on traumatic SCI, 80 adult male rats were randomly divided into seven groups: control, SCI (SCI induction by weight-drop method), MP (SCI induction, followed by administration of 30 mg/kg MP *via* the tail vein, once every other 6 hours, for five times), VC (SCI induction, followed by intraperitoneal administration of 100 mg/kg VC once a day, for 28 days), MP + VC (SCI induction, followed by administration of MP and VC as the former), BMMSCs (SCI induction, followed by injection of 3×10^6 BMMSCs at the injury site), and BMMSCs + VC (SCI induction, followed by BMMSCs injection and VC administration as the former). Locomotor recovery was assessed using the Basso Mouse Scale. Injured spinal cord tissue was evaluated using hematoxylin-eosin staining and immunohistochemical staining. Expression of transforming growth factor-beta, tumor necrosis factor-alpha, and matrix metalloproteinase-2 genes was determined using real-time quantitative PCR. BMMSCs intervention better promoted recovery of nerve function of rats with SCI, mitigated nerve cell damage, and decreased expression of transforming growth factor-beta, tumor necrosis factor-alpha, and matrix metalloproteinase-2 genes than MP and/or VC. More importantly, BMMSCs in combination with VC induced more obvious improvements. These results suggest that VC can enhance the neuroprotective effects of BMMSCs against SCI.

Key Words: nerve regeneration; spinal cord injury; vitamin C; methylprednisolone; bone marrow mesenchymal stem cells; locomotor; neural regeneration

Introduction

Spinal cord injury (SCI), which results from trauma or neurodegeneration, is a destructive injury. It often affects young and healthy individuals and debilitates them. It also creates a significant financial burden to the society (Song et al., 2014). Motor vehicle crashes and violence are the most common causes of traumatic spinal cord injury (TSCI), which is an irreversible condition that affects millions of people worldwide each year (Middleton et al., 2014). SCI has two phases: primary, followed by secondary injury. The primary injury is due to mechanical injury to cells, damages the spinal cord by interrupting its blood supply, and is usually severe at onset. Secondary injury includes neurogenic shock, respiratory failure (Lo et al., 2013), and bladder, bowel, cardiovascular, and sexual function disturbance (Harvey, 2016). Injuries to the spinal cord are ac-

companied by many problems which include neural cell death, inflammation, scar formation, and disturbance of neurovascular supply (Liu et al., 2013). The therapeutic effects of medical, surgical, and natural therapies are usually unsatisfactory (Li et al., 2015).

Corticosteroids contain naturally secreted steroid hormones which are produced from the adrenal cortex and synthetic hormones (Ngeow and Lim, 2016). Glucocorticoids are widely used in the treatment of many autoimmune and allergic diseases such as asthma and rheumatoid arthritis due to their well-authenticated anti-inflammatory effects (Peek et al., 2005). Methylprednisolone (MP), a synthetic glucocorticoid hormone, has anti-inflammatory, immunosuppressive, and anti-allergic effects and acts through inhibition of prostaglandins which are the main mediators of inflammation. So it is used

*Correspondence to:

Dina Sabry, M.D.,
dinasabry@kasralainy.edu.eg.

orcid:

0000-0002-6720-3385
(Dina Sabry)

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for treatment of inflammatory diseases (Jafari et al., 2016). MP, like other corticosteroids, is associated with potentially serious complications such as anaphylactic shock, cardiac arrhythmias, abnormal behavior (mood alteration, hyperactivity, and sleep disturbance), hyperglycemia, hypokalemia, and infections. Adverse effects of MP often occur in a dose-dependent manner (Sinha and Bagga, 2008; Jongen et al., 2016).

Plants can synthesize vitamin C (VC) for themselves, but humans and some other animals must obtain it from fruits and vegetables (Duan et al., 2016). VC is an essential dietary micronutrient that has many biological functions (Carr et al., 2016). VC is an important antioxidant against oxidative stress (Su et al., 2016) and was used successfully to treat many diseases as common cold (Lykkesfeldt and Poulsen, 2010), cancer (Aguilera et al., 2016; Frajese et al., 2016) and myocardial ischemia (El-Shitany and El-Desoky, 2016; Hao et al., 2016; Moradi-Arzelo et al., 2016). Deficiency of VC in diet leads to scurvy which is a serious disease (Carr et al., 2016).

Mesenchymal stem cells (MSCs) are adherent multipotent fibroblast-type stem cells which have the capacity of self-renewal and differentiation into mesodermal and ectodermal cells. MSCs are present in several tissues, including bone marrow, umbilical cord, and adipose tissue (Wang et al., 2016). Bone marrow-derived mesenchymal stem cells (BMMSCs) have anti-inflammatory, immunomodulatory, antiapoptotic, and antifibrotic effects through the secretion of bioactive trophic factors which make them suitable candidates for cell therapy. BMMSCs can survive for long periods after transplantation without inducing an immune response (Dhoke et al., 2016; Kim et al., 2016).

The present study was conducted to evaluate the therapeutic potential of MP, VC, and BMMSCs in experimentally induced traumatic SCI in rats.

Materials and Methods

Animals

Eighty healthy adult Albino male rats weighing 180 ± 30 g were bred and maintained in an air-conditioned animal house with specific pathogen-free conditions. Rats received a balanced diet with free access to water. All animal procedures were performed according to approved protocols of the Animal Committee of the Faculty of Medicine, Cairo University (Cuf/F/Sur/2014/28 (10/4/2014)). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH publication No. 85-23, revised 1996). They were kept under observation for one week before beginning of the experiment.

Experimental procedure

Rats were randomly divided into seven groups. Control group: twenty rats were subdivided equally into five subgroups: subgroup I (negative control rats) and subgroup II (sham operated controls), where rats were exposed to T₉₋₁₀ laminectomy without contusion injury and received no pharmacological treatment; subgroup III (MP vehicle control), where rats were injected with 0.5 mL of 0.9% saline via the caudal vein; subgroup IV (VC vehicle control), where rats were injected with 0.5 mL of 0.9% saline intraperitoneally; subgroup V, as

sham operated control with rats being injected once with 0.5 mL of phosphate buffered saline (PBS) locally at the site of laminectomy. SCI group: Ten rats were exposed to surgical induction of SCI. MP group: Ten rats were exposed to surgical induction of SCI and after one hour, MP sodium succinate in 0.9% saline (Solu-Medrol 1-gram vial, Pfizer, Boulevard de la Plaine, Ixelles, Belgium) was injected as 30 mg/kg intravenous bolus *via* rat tail vein followed by administration of MP for four subsequent times into a tail vein (30 mg/kg) at the 6-hour interval (Nash et al., 2002). VC group: Ten rats were exposed to surgical induction of SCI and after one hour, rats were injected intraperitoneally with VC (Cevaryl ampoule containing 500 mg VC) obtained from Memphis Company (8 EL-Sawah Square, EL-Amyria, Cairo, Egypt) at 100 mg/kg per day (Yan et al., 2014), and then further treated once a day for 28 days. MP + VC group: Ten rats were exposed to surgical induction of SCI and treated with combined MP and VC as in groups III and IV. BMMSCs group: Ten rats were exposed to surgical induction of SCI and received BMMSCs injection locally at the site of SCI at a single dose of 3×10^6 cells suspended in 0.5 mL phosphate buffered saline (PBS) (Attia et al., 2015). BMMSCs + VC group: Ten rats were exposed to surgical induction of SCI and treated with BMMSCs and VC as in VC and BMMSCs groups.

Induction of SCI

Surgical SCI induction was performed according to a previous study (Erbayraktar et al., 2013). In brief, aseptic T₉₋₁₀ laminectomy under anesthesia was performed. Spinal cord contusion was induced using a weight-drop apparatus, where a guided 10 g rod was dropped from a height of 12.5 mm onto the exposed dura mater, representing moderate SCI.

Preparation of BMMSCs

BMMSCs were flushed from rat tibia and fibula (these male healthy rats were not involved in experimental procedures) with phosphate buffered solution (PBS) containing 2 mL EDTA (Department of Biochemistry, Faculty of Medicine, Cairo University). 35 mL of the diluted sample was carefully layered using 15 mL Ficoll-Paque (Gibco-Invitrogen, Grand Island, NY, USA). Then, the mixture was centrifuged at $400 \times g$ for 35 minutes. The upper layer was aspirated, leaving the mononuclear cell layer undisturbed at the interphase. The mononuclear cell layer was aspirated, washed twice in PBS containing 2 mL EDTA, and then centrifuged for 10 minutes at $200 \times g$ at 10°C. The cell pellets were re-suspended in a final volume of 300 μ L of buffer. Isolated BMMSCs were cultured in a 25 mL culture flask in minimal essential medium supplemented with 15% fetal bovine serum (FBS) and incubated for 2 hours at 37°C in 5% CO₂ atmosphere. Adherent BMMSCs were cultured in minimal essential medium supplemented with 30% fetal bovine serum and 0.5% penicillin and streptomycin at 37°C in 5% CO₂ atmosphere (Yamazoe et al., 2007; Abdel Aziz et al., 2011). Cultured BMMSCs were confirmed using an inverted microscope: Leica DM IL LED with camera Leica DFC295 (Leica Microsystems CMS GmbH, Ernst-Leitz-Straße 17-37, Wetzlar, D-35578, Germany).

Labeling stem cells with GFP

BMMSCs were labeled with GFP (pAcGFP1-N1 Vector, Clon-

tech Laboratories, Inc. (USA) catalog # 632469) for in vivo tracing and observed in unstained spinal cord tissue cryosections (at T₉₋₁₀) using fluorescence microscope (Niki et al., 2004) (Leica Microsystems CMS GmbH, Ernst-Leitz-Straße, Wetzlar, D-35578, Germany).

Behavioral assessment

Hind limb locomotor recovery was assessed using the Basso Mouse Scale (BMS). The scale ranges from 0, indicating complete paralysis, to 9, indicating normal movement of the hind limbs (Basso et al., 2006). Rats of all groups were tested for hind limb functional deficits at 1, 7, 14, 21, and 28 days after SCI. All outcome measures were obtained in a blinded fashion by two investigators and averaged. The score was assessed at 1, 7, 14, 21 and 28 days postoperatively.

Histological and immunohistochemical staining

The fasted rats were anesthetized by 1.9% ether presented on a cotton ball or gauze pad for 5 to 10 minutes, and sacrificed by cervical decapitation four weeks after induction of SCI. Spinal cord tissue at the site of injury was excised. The specimens were fixed in 10% buffered formol saline, processed for 4–6 µm-thick paraffin sections, mounted on glass slides for hematoxylin-eosin staining (Bancroft and Layton, 2013). Other sections were mounted on positively charged slides for immunohistochemical staining (Jackson and Blythe, 2013).

Immunohistochemical staining was performed to detect nestin. Sections were incubated using primary antibody nestin (rat-401) at 4°C for 12 hours at 1–5 µg/mL. Then these sections were incubated with neurofilament (200 kDa) (lysine-serine-proline repeat) antibody (rabbit polyclonal, Catalog Number: AB1991, dilution: 1:1,000 using IHC-Tek™ antibody diluent (Cat# IW-1000 or IW-1001) at room temperature for 60 minutes to reduce background and unspecific staining. Serum blocking step was not needed.

Immunohistochemical reactions were performed using the horseradish peroxidase-labeled streptavidin-biotin method. Paraffin sections were mounted on positively charged slides. They were deparaffinized in xylene, rehydrated in descending grades of ethyl alcohol, and then immersed in 0.3% hydrogen peroxide in methyl alcohol to block endogenous peroxidase activity. The sections were washed in PBS and 10% normal rat serum was applied for 30 minutes to reduce nonspecific binding. After incubation for 1 hour with antiserum-containing primary antibody, the sections were counterstained with Mayer's hematoxylin, dehydrated in ascending grades of ethyl alcohol, cleared in xylene, and mounted in Canada balsam.

Morphometric study

The mean area percentage of nestin and NF-200 immunoreactive expression was quantified in five images from five non-overlapping fields of each rat using Image-Pro Plus program version 6.0 (Media Cybernetics Inc., Bethesda, Maryland, USA).

Real-time quantitative PCR (RT-qPCR)

Spinal cord tissue of all studied groups was homogenized. Total RNA was isolated with RNeasy Mini Kit (Qiagen, Hilden, Germany) and further analyzed for quantity and quality with

a dual beam spectrophotometer (Beckman Coulter, Fullerton, California, USA). For quantitative expression of transforming growth factor-beta (TGF-β), tumor necrosis factor-alpha (TNF-α), and matrix metalloproteinase-2 (MMP-2) genes, the following procedure was performed. 200 ng of the total RNA from each sample was used for cDNA synthesis by reverse transcription using High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems Inc., Foster City, CA, USA). The cDNA was subsequently amplified with the SYBR Green One-Step PCR Master Kit in a 48-well plate (Applied Biosystems Inc., Foster City, CA, USA) as follows: 10 minutes at 95°C for enzyme activation followed by 40 cycles of 15 seconds at 95°C, 20 seconds at 55°C, and 30 seconds at 72°C for the amplification step. Changes in the expression of each target gene were normalized relative to the mean critical threshold (CT) values of GAPDH housekeeping gene by the 2^{-ΔΔCt} method (Livak and Schmittgen, 2001). We used 1 µL of both primers specific for each target gene. Primer sequence and annealing temperature specific for each gene are demonstrated in **Table 1**.

Statistical analysis

All data collected from the experiment were recorded and analyzed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) with *post hoc* Scheffe's test was used to compare differences among the groups. In each test, the data were expressed as the mean ± standard deviation (SD) and differences were considered to be significant at *P* < 0.01.

Results

Behavior changes in SCI rats

All data of BMS score for all groups and at all time points are shown in **Table 2**. Rats in the control group showed normal rat locomotor function with a BMS score of 9 at all time points. Rats in other groups did not show any recovery at 1 day. Slight recovery in rats from the SCI group began from day 7 and continued until day 28. There were no significant differences in BMS score at all time points between SCI group and MP and VC groups. BMS score increased significantly in the MP + VC group at 21 and 28 days, in the BMMSCs group at 14, 21 and 28 days, and in the BMMSCs + VC group at 7, 14, 21 and 28 days (*P* < 0.01).

BMMSCs identification in culture and tracking

BMMSCs were identified in culture under an inverted microscope as spindle shaped cells between rounded cells (**Figure 1A**). Locally injected BMMSCs labeled with GFP were observed in spinal cord tissue using a fluorescent microscope (**Figure 1B**).

Hematoxylin-eosin staining

In the control group, all subgroups showed nearly the same histological images. They showed central gray matter and peripheral white matter. The white matter contained thick fibers and neuroglial cells. The gray matter contained nerve fibers, nerve cells, and neuroglial cells (**Figure 2A**). A higher magnification of the gray matter showed a number of multipolar cells with central nuclei and multiple Nissl's granules in their cytoplasm. Neuroglial cells could be seen (**Figure 2B**). SCI

Table 1 Primer sequence and specific for each gene

Target gene	Primer sequence: 5'–3'	Gene bank accession number
TGF-β	Forward: TGC GCC TGC AGA GAT TCA AG Reverse: AGG TAA CGC CAG GAA TTG TTG CTA	NM021578.2
TNF-α	Forward: AGA TGT AGG TGT ACA CAG CC Reverse: GTC ACG CGG TTG TCG AAA CT	NM041474.1
MMP-2	Forward: CTA TTC TGT CAG CAC TTT GG Reverse: CAG ACT TTG GTT CTC CAA CTT	NM031054.2
GAPDH	Forward: CAC CCT GTT GCT GTA GCC ATA TTC Reverse: GAC ATC AAG AAG GTG GTG AAG CAG	NG028301.2

Table 2 Basso Mouse Scale (BMS) score at 1, 7, 14, 21, and 28 days after SCI in all groups

	Control	SCI	MP	VC	MP + VC	BMMSCs	BMMSCs + VC
1 day	9±0	0	0	0	0	0	0
7 days	9±0 ^{bcdefg}	0.5±0.53 ^{ag}	0.8±0.42 ^{ag}	0.8±0.42 ^{ag}	1.3±0.68 ^a	1.0±0.47 ^a	1.8±0.42 ^{abcd}
14 days	9±0 ^{bcdefg}	0.5±0.53 ^{afg}	1.0±0.67 ^{afg}	1.2±0.63 ^{afg}	1.5±0.53 ^{afg}	2.6±0.97 ^{abcdg}	4.6±1.08 ^{abcdef}
21 days	9±0 ^{bcdefg}	0.8±0.42 ^{afg}	1.3±0.68 ^{afg}	1.5±0.53 ^{afg}	2.4±0.52 ^{abcf}	5.2±0.63 ^{abcde}	5.9±0.74 ^{abcde}
28 days	9±0 ^{bcdef}	1.1±0.57 ^{afg}	1.3±0.48 ^{afg}	2.1±0.74 ^{afg}	2.9±0.74 ^{abcf}	6.3±0.82 ^{abcdeg}	7.9±0.88 ^{bcdef}

Data are expressed as the mean ± SD from $n = 20$ rats in the control group and $n = 10$ rats from other groups. ^a $P < 0.01$, vs. control group; ^b $P < 0.01$, vs. SCI group; ^c $P < 0.01$, vs. MP group; ^d $P < 0.01$, vs. VC group; ^e $P < 0.01$, vs. MP + VC group; ^f $P < 0.01$, vs. BMMSCs group; ^g $P < 0.01$, vs. BMMSCs + VC group (one-way analysis of variance and *post hoc* Scheffe's test). SCI: Spinal cord injury; MP: methylprednisolone; VC: vitamin C; BMMSCs: bone marrow mesenchymal stem cells.

Table 3 Nestin- and neurofilament 200 (NF200)-immunoreactive area (%) in the spinal cord of rats

	Control	SCI	MP	VC	MP + VC	BMMSCs	BMMSCs + VC
Nestin	0	0	0	0.13±0.027	0.31±0.095	2.34±0.262	4.07±0.539
NF200	29.57±0.774 ^{bcde}	7.77±0.623 ^{adefg}	10.52±4.932 ^{adefg}	18.85±2.360 ^{abcf}	19.38±1.586 ^{abcf}	27.31±1.099 ^{bcde}	30.60±0.431 ^{bcde}

Data are expressed as the mean ± SD from $n = 20$ rats in the control group and $n = 10$ rats from other groups. ^a $P < 0.01$, vs. control group; ^b $P < 0.01$, vs. SCI group; ^c $P < 0.01$, vs. MP group; ^d $P < 0.01$, vs. VC group; ^e $P < 0.01$, vs. MP + VC group; ^f $P < 0.01$, vs. BMMSCs group; ^g $P < 0.01$, vs. BMMSCs + VC group (one-way analysis of variance and *post hoc* Scheffe's test). SCI: Spinal cord injury; MP: methylprednisolone; VC: vitamin C; BMMSCs: bone marrow mesenchymal stem cells.

Table 4 TGF-β, TNF-α and MMP-2 gene expression in the spinal cord of rats

	Control	SCI	MP	VC	MP + VC	BMMSCs	BMMSCs + VC
TGF-β	1.1±0 ^{bcdefg}	2.97±0.527 ^{ag}	0.8±0.422 ^{ag}	0.8±0.422 ^{ag}	1.3±0.675 ^a	1±0.471 ^a	1.8±0.422 ^{abcd}
TNF-α	9±0 ^{bcdefg}	0.5±0.527 ^{afg}	1.0±0.667 ^{afg}	1.2±0.632 ^{afg}	1.5±0.527 ^{afg}	2.6±0.966 ^{abcdg}	4.6±1.075 ^{abcdef}
MMP-2	9±0 ^{bcdef}	1.1±0.568 ^{afg}	1.3±0.483 ^{afg}	2.1±0.738 ^{afg}	2.9±0.738 ^{abcf}	6.3±0.823 ^{abcdeg}	7.9±0.876 ^{bcdef}

Data are expressed as the mean ± SD from $n = 20$ rats in the control group and $n = 10$ rats from other groups. Relative target mRNA expression was normalized to housekeeping gene GAPDH. ^a $P < 0.01$, vs. control group; ^b $P < 0.01$, vs. SCI group; ^c $P < 0.01$, vs. MP group; ^d $P < 0.01$, vs. VC group; ^e $P < 0.01$, vs. MP + VC group; ^f $P < 0.01$, vs. BMMSCs group; ^g $P < 0.01$, vs. BMMSCs + VC group (one-way analysis of variance and *post hoc* Scheffe's test). SCI: Spinal cord injury; MP: methylprednisolone; VC: vitamin C; BMMSCs: bone marrow mesenchymal stem cells; TGF-β: Transforming growth factor-beta; TNF-α: tumor necrosis factor-alpha; MMP-2: matrix metalloproteinase-2.

group showed areas of complete destruction of nerve cells with marked vacuolation, congested dilated blood vessels and excess neuroglial cells (gliosis). Few apparently normal neurons with wide perineural space and some dark disfigured neurons were noticed (Figure 2C). MP group showed areas of complete destroyed neurons. Some neurons appeared normal and others appeared disfigured with wide perineural space. Blood vessels appeared slightly dilated and congested. There were many neuroglial cells. Thick fibers in gray and white matters were noticed (Figure 2D). VC group showed few apparently normal neurons

but most of the neurons were disfigured with wide perineural space. Blood vessels appeared dilated and congested. There were many neuroglial cells (Figure 2E). MP + VC group showed some apparently normal neurons, and others were disfigured neurons with slightly wide perineural space. Blood vessels appeared slightly congested. There were many neuroglial cells (Figure 2F). BMMSCs group showed apparently normal neurons with central nuclei and Nissl's granules in their cytoplasm, many neuroglial cells and small blood vessels. Few disfigured neurons and wide perineural space were observed (Figure 2G).

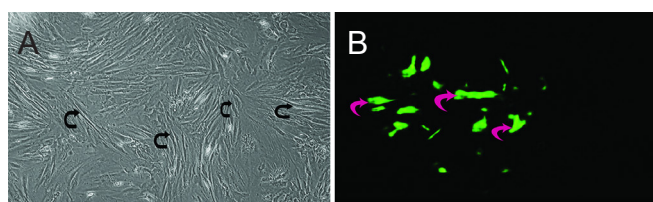


Figure 1 Identification of cultured rat bone marrow mesenchymal stem cells (BMMSCs).

(A) Many spindle shaped stem cells (arrows) from primary culture at 14 days (inverted microscope). (B) BMMSCs labeled with green fluorescent protein (arrows) from stem cell groups (BMMSCs and BMMSCs + VC groups) (fluorescent microscope). Original magnification 400× in A and B. VC: Vitamin C.

BMMSCs + VC group showed a similar image to the control group. There were a number of apparently normal neurons with central nuclei, multiple Nissl's granules in their cytoplasm, many neuroglial cells and small blood vessels (**Figure 2H**).

Nestin immunoreactivity

Nestin, a class VI intermediate filament protein is expressed in adult neural progenitor cells (NPCs). Nestin-immunoreactive cells showed brown cytoplasmic staining. Control, SCI, and MP groups showed negative nestin expression (**Figure 3A–C**). VC and MP + VC groups showed minimal nestin-immunoreactive expression (**Figure 3D, E**). BMMSCs and BMMSCs + VC groups showed marked nestin-immunoreactive expression (**Figure 3F, G**).

Neurofilament 200 (NF200) immunoreactivity

NF200 was expressed in somal and axonal membranes as brown staining particles. Control group showed apparent NF200 immunoreactivity (**Figure 4A**). SCI group showed marked depletion in NF immunoreactivity (**Figure 4B**). MP, VC, and MP + VC groups showed increased NF immunoreactivity compared with SCI group (**Figure 4C–E**). NF200 immunoreactivity was obviously increased in the BMMSCs and BMMSCs + VC groups than in the SCI group (**Figure 4F, G**).

Nestin and NF200 immunoreactivity in all groups is shown in **Table 3** and **Figures 3H, 4H**. NF200 immunoreactivity in the VC, MP + VC, BMMSCs, and BMMSCs + VC groups was significantly higher than that in the SCI group ($P < 0.01$).

Quantitative gene expression

Gene expression of TGF- β (pro-fibrotic and inflammatory marker), TNF- α (inflammatory marker) and MMP-2 (fibrosis marker) in all groups is shown in **Table 4**. The expression levels of TGF- β , TNF- α and MMP-2 gene were not significantly decreased in the MP, VC, and MP + VC groups than in the SCI group. However, the expression level of these three genes was significantly decreased in the BMMSCs, and BMMSCs + VC groups ($P < 0.01$).

Discussion

Traumatic SCI is a serious and destructive condition which leads to loss of function below the injury site, with considerable effects on the quality and expectancy of life. There is no ideal or effective cure for SCI at this time (Lee et al., 2016).

The SCI group in the present study demonstrated slight increase in BMS score at 7 days which continued until 28 days,

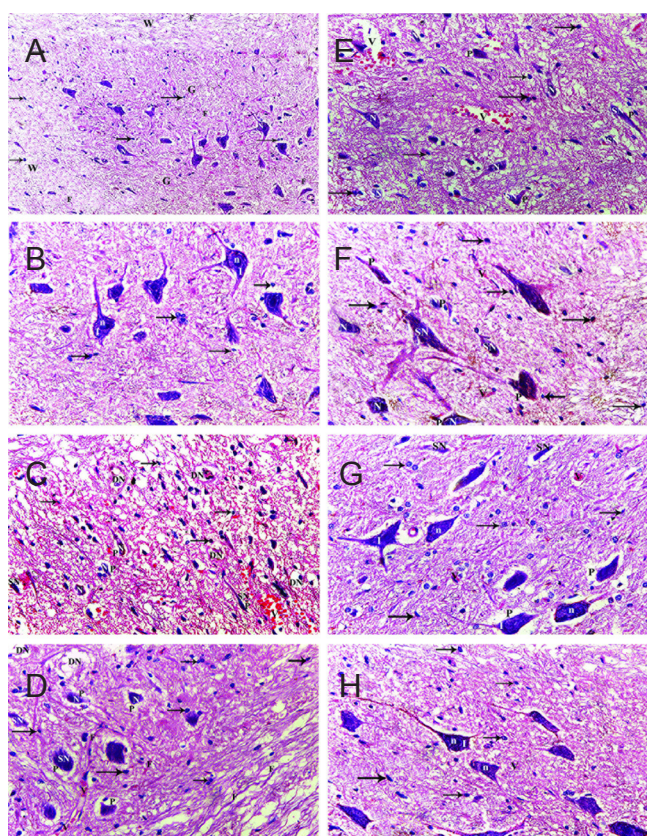


Figure 2 Representative micrographs of hematoxylin-eosin-stained rat spinal cord tissue sections.

(A) Control group: showing central gray matter (G) and peripheral white matter (W). The white matter contained nerve fibers (F) and neuroglia cells (arrow). The gray matter contained nerve fibers (F), nerve cells (N), and neuroglial cells (arrow). (B) In higher magnification of (A), gray matter shows apparently normal neurons with central nuclei (n) and Nissl's granules (I) in their cytoplasm and neuroglial cells (arrow). (C) Spinal cord injury (SCI) group: showing areas of complete destruction of nerve cells with marked vacuolation (DN), congested dilated blood vessels (V) and excess neuroglial cells (arrow). Few apparently normal neurons (N) with wide perineural space (P) and some dark disfigured neurons (SN) are observed. (D) Methylprednisolone (MP) group: showing few areas of complete destroyed neurons (DN), some neurons appear normal (N) and others appear disfigured (SN) with wide perineural space (P). Blood vessels appear slightly dilated and congested (V). There are multiple neuroglial cells (arrow) and thick nerve fibers (F) in gray and white matters. (E) Vitamin C (VC) group: showing few apparently normal neurons (N), many disfigured neurons (SN) with wide perineural space (P). Blood vessels appear dilated and congested (V). There are multiple neuroglial cells (arrow). (F) MP + VC group: showing some apparently normal neurons (N) and other disfigured (SN) neurons with slightly wide perineural space (P). Blood vessels (V) appeared slightly congested. There were multiple neuroglial cells (arrow). (G) Bone marrow mesenchymal stem cells (BMMSCs) group: showing apparently normal neurons (N) with central nuclei and Nissl's granules (I) in their cytoplasm, many neuroglial cells (arrow) and small blood vessels (V). Few disfigured neurons and wide perineural space (P) are observed. (H) BMMSCs + VC group: showing apparently normal neurons with central nuclei (n), and multiple Nissl's granules (I) in their cytoplasm, many neuroglial cells (arrow) and small blood vessels (V). Original magnification, A: 200×; B–H: 400×.

areas of complete destruction of nerve cells with excess neuroglial cells (gliosis), negative nestin expression, marked depletion in NF expression, and high TGF- β , TNF- α , and MMP-2 gene expression. In accordance with these results, an early study (Pajoohesh-Ganji et al., 2010) revealed that BMS scores

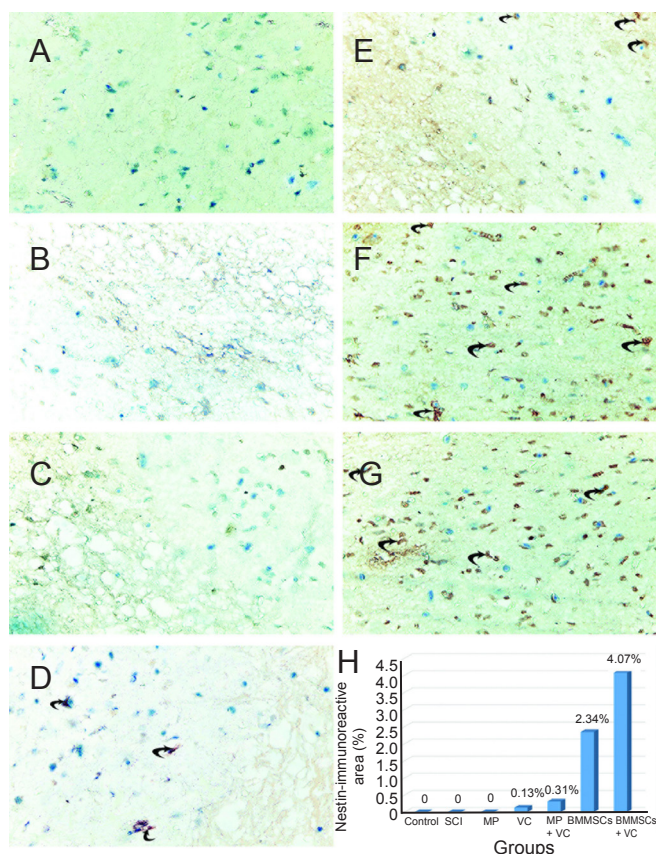


Figure 3 Nestin immunoreactivity in spinal cord tissue section.

(A–G) Immunohistochemical staining for nestin (original magnification, 400 \times). No nestin immunoreactivity was observed in the control (A), SCI (B), and MP (C) groups. Weak nestin immunoreactivity (arrows) was observed in the VC (D) and MP + VC groups (E). Obvious nestin immunoreactivity was observed in the BMMSCs (F) and BMMSCs + VC groups (G). (H) Quantification of nestin immunoreactivity. All data are expressed as the mean \pm SD. SCI: Spinal cord injury; MP: methylprednisolone; VC: vitamin C; BMMSCs: bone marrow mesenchymal stem cells.

slightly increased at 7 days and stabilized until day 42. There was acute functional loss one day after the injury followed by progressively restricted recovery. Several studies (Yan et al., 2016; Zhang et al., 2016; Zhou et al., 2016) reported that SCI rats showed posttraumatic progressive neuron cell loss, glial proliferation, and degeneration of the injured spinal cord. The levels of oxidative stress and apoptosis are believed to be two of the most serious factors leading to nervous tissue destruction. Likewise, Jiang et al. (2014) stated that real-time PCR showed a significant increase in TNF- α in the spinal cord after injury. Shibuya et al. (2002) reported that in rats with SCI, nestin immunoreactivity was noticed at the pial surface surrounding the spinal cord and at the lesion site. In spite of the fact that nestin expression was negligible at 24 hours, and it expanded definitely at 1 and 4 weeks after injury. However, some scholars (Namiiki and Tator, 1999; Cizkova et al., 2009) reported that nestin expression was detected around the central canal 4 days after SCI, increased and surrounded the lesion site, and then decreased by day 7 to return to its basal level by day 14; thus only limited nestin labeling around lesion site could be seen. Also, one recent study (Geng et al., 2015) showed that after SCI,

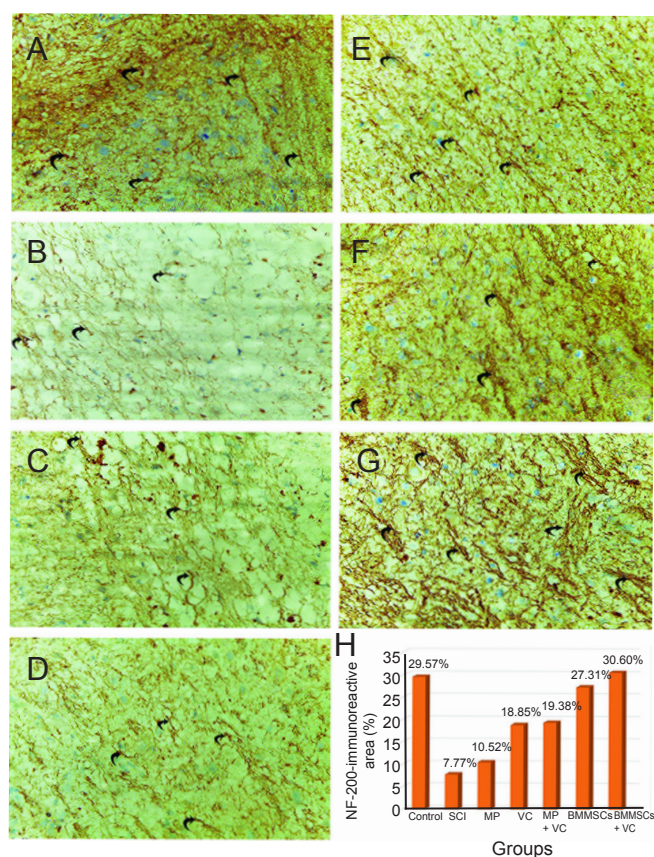


Figure 4 Neurofilament 200 (NF200) immunoreactivity in spinal cord tissue section.

(A–G) Immunohistochemical staining for NF200 (original magnification, 400 \times). Strong NF200 immunoreactivity (arrow) was observed in the control (A), BMMSCs (F), BMMSCs + VC (G) groups. Moderate NF200 immunoreactivity (arrows) was observed in the SCI (B), MP (C), VC (D), and MP + VC (E) groups. (H) Quantification of NF200 immunoreactivity. All data are expressed as the mean \pm SD. SCI: Spinal cord injury; MP: methylprednisolone; VC: vitamin C; BMMSCs: bone marrow mesenchymal stem cells.

nestin protein expression was suppressed, and nestin-immunoreactive cells were limited and localized to the ependymal canal. In the present study, no inflammatory cells (neutrophilic) infiltrated in the hematoxylin-eosin-stained sections. Fleming et al. (2006) reported that, in rats, neutrophilic infiltrations occurred early (4–6 hours) after SCI and disappeared within 5 days, and macrophages (derived from blood-borne monocytes and resident microglia) penetrated the lesion 2 days after SCI in rats and continued for weeks to months.

Our results showed that in the MP group, BMS score was not significantly increased at all time points compared with the SCI group, some neurons completely destroyed, and the other neurons were disfigured with wide perineural space and negative nestin expression. In addition, there were no significant differences in NF200 expression and TGF- β , TNF- α , and MMP-2 gene expression between MP and SCI groups. All these findings suggest that MP exhibits slight, but not significant therapeutic effects on SCI. These results are in consistent with those reported by some scholars (Lankhorst et al., 2000; Marcon et al., 2010; Harrop, 2014) who demonstrated that MP-treated rats did not show significant locomotor recovery

after SCI. Moreover, Is et al. (2006) reported that MP-treated rats showed no improvement in the histopathological findings compared with rats in the SCI group except for a mild decrease in edema formation in spinal cord tissue. However, some scholars (Hugenholtz, 2003; Sharma et al., 2004) reported that MP was effective in enhancing locomotor and histological recovery and reducing edema when given early after SCI. Others (Evaniew et al., 2015; Sámano et al., 2016) attributed this controversy to the variation of the studies in MP dose timing and duration, species and age of animals, degree and extent of SCI, and the methods of evaluations and outcome analysis. Several studies (Sayer et al., 2006; Hurlbert et al., 2013; Lim and Choi, 2017) reported that there is no convenient medical evidence of any class to warrant the utilization of MP for acute SCI. MP administration is associated with many serious complications and even death and should not be routinely used in the treatment of patients with acute SCI as it is not recommended for use in acute SCI according to the USA Food and Drug Administration.

Our results showed that in the VC group, histological manifestation was slightly improved, there was minimal nestin expression, and NF200 expression was significantly increased compared with the SCI group. This is consistent with the findings reported by Cristante et al. (2009) that VC only relieved histopathological injury, but not neurological performance after SCI. However, Yan et al. (2014) reported that a higher dose of VC (200 mg/kg per day) after SCI significantly diminished the secondary damage-induced tissue necrosis and enhanced the behavioral function of rats. This impact of VC on SCI was attributed to its antioxidant and oxygen free radical's scavenger impact as the pathologic course of spinal cord contusion incorporates primary and secondary mechanisms of damage and the oxygen free radicals were recommended as essential factors in secondary injury (Jia et al., 2012; Chen et al., 2014; Fatima et al., 2015).

Our results showed that in the MP + VC group, histological manifestation was greatly improved compared with the MP or VC group alone. In the MP + VC group, there was minimal nestin expression, BMS scores increased significantly at 21 and 28 days only, and NF200 expression was significantly increased compared with the SCI group. Is et al. (2016) revealed that MP or antioxidant alone had a mild beneficial effect but the combination was more effective and produced an additive effect on improving histopathological and locomotor changes after SCI. Recent studies (Anwar et al., 2016; Li et al., 2016) demonstrated that since the wide range of pathogenic procedures is included in SCI, it is improbable that treatment with a single agent will lead to maximum recovery and combined multiple agents should be applied in treatment strategies.

In this study, BMMSCs were injected locally into the lesion site in SCI rats as some previous studies (Takahashi et al., 2011; Elawady et al., 2016; Liu et al., 2016) have demonstrated that direct injection of stem cells into a spinal cord lesion site is more successful than intravenous injection in functional recovery of injured spinal cord in rats. Amemori et al. (2015) showed that direct injection of mesenchymal stem cells into a spinal cord compression lesion promoted locomotor recovery. In the BMMSCs group in the current study, locomotor activity was obviously increased, as confirmed by significant increase in BMS score at 14, 21 and 28 days, compared with

the SCI group. In the BMMSCs group, there was much nestin expression, NF200 expression was significantly increased, and TGF- β , TNF- α , and MMP-2 gene expression was significantly decreased compared with the SCI group. Several previous studies (Tohda and Kuboyama, 2011; Park et al., 2012; Oliveri et al., 2014; Kim et al., 2015; Anna et al., 2017) demonstrated that transplantation of mesenchymal stem cells into SCI models promoted functional recovery after SCI, and the optimal dose, timing, and route of BMMSCs administration were important factors for achieving therapeutic results. Saini et al. (2016) reported that transplantation of BMMSCs in rats with SCI increased NF200 expression and promoted functional recovery. Xie et al. (2015) reported that BMMSCs exhibited positive nestin expression. The mechanisms underlying recovery include differentiation of BMMSCs into neuronal cells which express neural cell marker. BMMSCs are likewise ready to regulate the injured microenvironment of the central nervous system and to enhance healing by paracrine as they secrete anti-apoptotic and anti-inflammatory factors and trophic molecules. These trophic molecules can support axonal growth, immunomodulation, to enhance angiogenesis, remyelination, and to protect against apoptotic cell death (Quertainmont et al., 2012; Zhu et al., 2013; Kakabadze et al., 2016; Uchida et al., 2016).

Combined therapies for the treatment of SCI are more effective than monotherapy due to the complexity of SCI (Butenschön et al., 2016; Jin et al., 2016). Low efficacy of stem cell transplantation was attributed to the unsuitable pathological microenvironment at the injured sites as oxidative stress, inflammation, and using antioxidants result in an increase in stem cell survival and efficacy after transplantation (Saparov et al., 2013; Yang et al., 2015). Likewise, more improvement in all parameters were observed in the BMMSCS + VC group than in the BMMSCs group.

Taken together, MP or VC alone had a little therapeutic effect on traumatic SCI in rats, and their combination was capable of improving the locomotor activity and had some beneficial effects on modulating the histopathological manifestation of rats with SCI. However, BMMSCs transplantation had an effective role in functional and histological reconstruction of the spinal cord in rats after injury and BMMSCs in combination with VC was more therapeutically effective.

Author contributions: MME and MzAE designed the study and performed data analysis. NS, MYS, DS and AE were responsible for histological and molecular assessment of the work. MoAE was in charge of statistical analysis. AS and AHHE were responsible for surgical procedure of the experiment. NI assessed physiological and molecular procedures. All authors contributed to writing of the paper and approved the final version of this paper.

Conflicts of interest: None declared.

Research ethics: The study procedures were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH publication No. 85-23, revised 1996) and were approved by the Animal Committee of the Faculty of Medicine, Cairo University (Cuf/F/Sur/2014/28 (10/4/2014)).

Data sharing statement: Datasets analyzed during the current study are available from the corresponding author on reasonable request.

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